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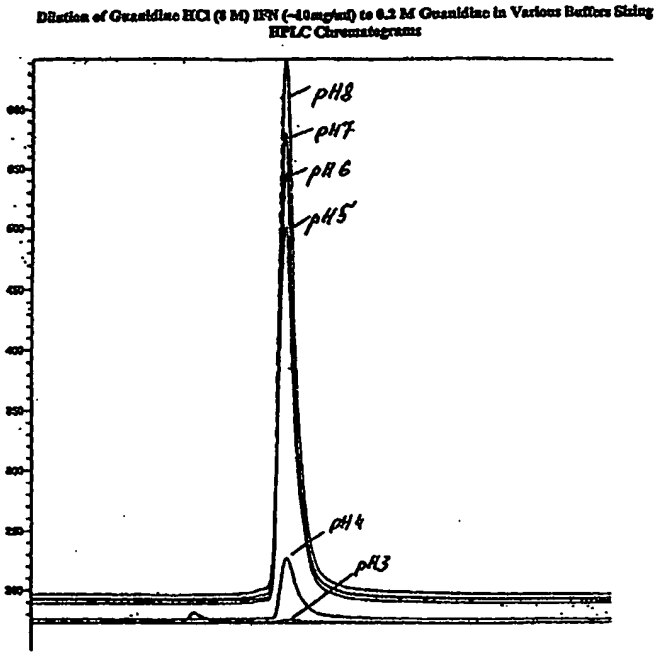
(54) Title: METHODS OF PROTEIN PURIFICATION AND RECOVERY



(57) Abstract: Improved methods for purification and recovery of interferon-beta (IFN-β) and compositions comprising substantially monomeric IFN-β are provided. In one purification method, substantially purified IFN-β or variant thereof is precipitated and then dissolved in a guanidine hydrochloride (HC1) solution. Renaturation of the protein occurs by dilution with a suitable buffer. A similar purification method absent the precipitation step is also provided. Following renaturation of the IFN-β, residual guanidine HC1 is removed by diafiltration or dialysis with a pharmaceutically acceptable buffer to prepare pharmaceutical compositions comprising substantially monomeric IFN-β.

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SUMMARY OF THE INVENTION

Improved methods useful in the preparation of pharmaceutical formulations of IFN-β are provided. The methods provide monomeric, liquid pharmaceutical compositions comprising IFN-β. The methods include conditions that enhance refolding of the protein during the recovery process.

To achieve the foregoing and other objects and in accordance with the purpose of the present invention as embodied and broadly described herein, the present invention provides improved methods for the purification and recovery of IFN-β. In one embodiment, the improved method comprises preparing a solution comprising IFN-β, isolating a pool of substantially purified IFN-β from this solution, precipitating the purified IFN-β from this pool using an alcohol, and dissolving the precipitated IFN-β into guanidine hydrochloride to form a solution comprising resolubilized denatured IFN-β. This solution comprising resolubilized denatured IFN-β is then diluted into an appropriate first buffer to obtain a solution comprising resolubilized renatured IFN-β. The resulting solution is then diafiltered or dialyzed into a buffer suitable for pharmaceutical purposes. This last step removes residual guanidine hydrochloride, yielding a pharmaceutical formulation comprising substantially monomeric IFN-β suitable for parenteral administration.

In another embodiment, the improved method of purification and recovery of IFN-β comprises obtaining a sample of substantially purified IFN-β and mixing this sample with guanidine hydrochloride to form a solution comprising solubilized denatured IFN-β. This solution comprising solubilized denatured IFN-β is then diluted into an appropriate first buffer to obtain a solution comprising solubilized renatured IFN-β. The resulting solubilized renatured IFN-β solution is then diafiltered or dialyzed into a buffer suitable for pharmaceutical purposes. As noted above, this last step removes the residual guanidine hydrochloride, yielding a pharmaceutical formulation comprising substantially monomeric IFN-β suitable for parenteral administration.

Another aspect of the present invention deals with an improved process for the recovery of microbially produced IFN-β. Using the methods of the invention, it is possible to prepare IFN-β pharmaceutical formulations that are free or virtually free of SDS (less than 10 micrograms SDS per milligram of IFN-β). Another aspect of the present invention is that substances such as human serum albumin (HSA) are not

monomeric form, as opposed to its aggregated form, preferably at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, more preferably at least about 90% or more of the IFN-β in its monomeric form.

In one embodiment, the composition comprising substantially monomeric IFN-β is prepared by precipitating substantially purified IFN-β from solution, resuspending the precipitate by dissolution in guanidine hydrochloride (HCl), removing any residual SDS by filtration where the initial IFN-β sample comprises SDS, and then renaturing the IFN-β by dilution of the resulting guanidine HCl-IFN-β solution with an appropriate buffer solution. By "substantially purified" is intended the IFN-β in the starting material is substantially or essentially free from components that normally accompany or interact with the protein as found in its naturally occurring environment, i.e., a native cell, or host cell in the case of recombinantly produced IFN-β. An IFN-β polypeptide that is substantially free of cellular material includes preparations of protein having less than about 30%, 25%, 20%, 15%, 10%, 5%, or 1% (by dry weight) of contaminating protein. When the IFN-β polypeptide or biologically active variant thereof is recombinantly produced, preferably culture medium represents less than about 30%, 25%, 20%, 15%, 10%, 5%, or 1% (by dry weight) of chemical precursors or non-protein-of-interest chemicals. Thus, "substantially purified" IFN-β for use in the methods of the present invention is said to have a purity level of at least about 70%, preferably a purity level of at least about 75%, 80%, 85%, more preferably a purity level of at least about 90% or greater as determined by SDS/PAGE analysis.

In another embodiment, the composition comprising substantially monomeric IFN-β is prepared in the absence of the precipitation step noted above. In this manner, a sample comprising substantially purified IFN-β is mixed with guanidine HCl to obtain a solution comprising solubilized denatured IFN-β; the IFN-β is then renatured by dilution of the resulting guanidine HCl-IFN-β solution with an appropriate buffer. The ramifications of these preparation steps are the basis for the compositions comprising substantially monomeric IFN-β and methods of the present invention for preparing injectable formulations comprising substantially monomeric IFN-β that are useful for IFN-β therapy directed to IFN-β-responsive diseases.

The skilled artisan will appreciate that additional changes can be introduced by mutation into the nucleotide sequences encoding IFN-β, thereby leading to changes in the IFN-β amino acid sequence, without altering the biological activity of the interferon. Thus, an isolated nucleic acid molecule encoding an IFN-β variant having a sequence that differs from the amino acid sequence for the native IFN-β can be created by introducing one or more nucleotide substitutions, additions, or deletions into the corresponding nucleotide sequence encoding the native IFN-β, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded IFN-β. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Such IFN-β variants are also encompassed by the present invention.

For example, conservative amino acid substitutions may be made at one or more predicted, preferably nonessential amino acid residues. A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of IFN-β without altering its biological activity, whereas an "essential" amino acid residue is required for biological activity. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Such substitutions would not be made for conserved amino acid residues, or for amino acid residues residing within a conserved motif.

Alternatively, variant IFN-β nucleotide sequences can be made by introducing mutations randomly along all or part of an IFN-β coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for IFN-β biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly, and the activity of the protein can be determined using standard assay techniques described herein.

detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, gapped BLAST, or PSI-BLAST programs, the default parameters can be used. *See* the website for ncbi.nlm.nih.gov. Also see the ALIGN program (Dayhoff (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3, National Biomedical Research Foundation, Washington, D.C.) and programs in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, Wisconsin), for example, the GAP program, where default parameters of the programs are utilized.

When considering percentage of amino acid sequence identity, some amino acid residue positions may differ as a result of conservative amino acid substitutions, which do not affect properties of protein function. In these instances, percent sequence identity may be adjusted upwards to account for the similarity in conservatively substituted amino acids. Such adjustments are well known in the art. See, for example, Myers and Miller (1988) *Comput. Appl. Biosci.* 4:11-17.

Biologically active variants of IFN-β encompassed by the invention should retain IFN-β activities, particularly the ability to bind to IFN-β receptors. The biological activity of IFN-β variants can be measured by any method known in the art. Examples of such assays can be found in Fellous *et al.* (1982) *Proc. Natl. Acad. Sci USA* 79:3082-3086; Czerniecki *et al.* (1984) *J. Virol.* 49(2):490-496; Mark *et al.* (1984) *Proc. Natl Acad. Sci. USA* 81:5662-5666; Branca *et al.* (1981) *Nature* 277:221-223; Williams *et al.* (1979) *Nature* 282:582-586; Herberman *et al.* (1979) *Nature* 277:221-223; and Anderson *et al.* (1982) *J. Biol. Chem.* 257(19):11301-11304.

Non-limiting examples of IFN-β polypeptides and IFN-β variant polypeptides encompassed by the invention are set forth in Nagata *et al.* (1980) *Nature* 284:316-320; Goeddel *et al.* (1980) *Nature* 287:411-416; Yelverton *et al.* (1981) *Nucleic Acids Res.* 9:731-741; Streuli *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2848-2852; EP028033B1, and EP109748B1. See also U.S. Patent Nos. 4,518,584; 4,569,908; 4,588,585; 4,738,844; 4,753,795; 4,769,233; 4,793,995; 4,914,033; 4,959,314; 5,545,723; and 5,814,485. These disclosures are herein incorporated by reference. These citations also provide guidance regarding residues and regions of the IFN-β polypeptide that can be altered without the loss of biological activity.

Meinhofer, Vol. 2 (Academic Press, New York, 1980), pp. 3-254, discussing solid-phase peptide synthesis techniques; and Bodansky (1984) *Principles of Peptide Synthesis* (Springer-Verlag, Berlin) and Gross and Meinhofer, eds. (1980) *The Peptides: Analysis, Synthesis, Biology*, Vol. 1 (Academic Press, New York), discussing classical solution synthesis. IFN-β can also be chemically prepared by the method of simultaneous multiple peptide synthesis. See, for example, Houghten (1984) *Proc. Natl. Acad. Sci. USA* 82:5131-5135; and U.S. Patent No. 4,631,211.

Preparation of the compositions comprising substantially monomeric IFN-β disclosed herein is preferably carried out in accordance with one of the two improved purification methods of the present invention. The first of these purification methods comprises three basic steps: (1) precipitation of IFN-β from a solution comprising substantially purified IFN-β; (2) dissolution of the IFN-β precipitate in guanidine hydrochloride (HCl) to achieve resolubilization of the IFN-β; and (3) renaturation of the IFN-β, preferably via dilution or dialysis using an acceptable buffer. This purification method produces IFN-β that is soluble, stable, and in substantially monomeric form. The resulting composition can be formulated as a pharmaceutical composition by further diafiltration or dialysis of this composition with a pharmaceutically acceptable buffer. This final step removes residual guanidine HCl from the solution comprising renatured IFN-β and provides for a formulation having a pH that is acceptable for parenteral administration.

Using this purification method of the invention, a precipitate of IFN-β is first prepared by precipitating substantially purified IFN-β from a solution. Precipitation is accomplished by reducing the solubility of IFN-β. Reduction of IFN-β solubility and precipitation of IFN-β may be achieved with the use of an alcohol, for example an aliphatic alcohol such as ethanol. For some proteins, precipitation results from a denaturation and/or aggregation reaction that is irreversible, leading to protein inactivation, but in the case of the precipitated IFN-β of the present invention, the precipitation reaction is reversible. Thus, the soluble IFN-β recovered in the subsequent steps of this purification method retains its biological activity.

The resulting precipitate is then dissolved in guanidine HCl to obtain a solution comprising resolubilized denatured IFN-β and guanidine HCl. In those instances where the substantially purified IFN-β has been obtained using an initial

residual guanidine HCl remaining in the resolubilized renatured IFN-β solution is about 1.6 M or less, more preferably about 0.8 M or less.

The resulting resolubilized renatured IFN-β solution comprises the IFN-β substantially in its monomeric form (i.e., greater than about 51% is monomeric). In addition, the resolubilized renatured IFN-β solution comprises a residual amount of guanidine HCl. This composition can be utilized for preparation of pharmaceutical formulations that are suitable for parenteral administration. In this manner, the residual guanidine HCl solubility enhancer can be removed from the resolubilized renatured IFN-β solution by dialysis or diafiltration of this solution with a pharmaceutically acceptable buffer. By "removal of residual guanidine HCl" is intended the pharmaceutical formulation comprising substantially monomeric IFN-β prepared using the steps of this purification method comprises guanidine HCl at a concentration of 10 mM or less, preferably 5 mM or less. Any pharmaceutically acceptable buffer can be used to make the pharmaceutical formulation so long as the IFN-β remains solubilized and substantially in its monomeric form. In one embodiment, the pharmaceutically acceptable buffer comprises arginine or sodium chloride in an amount sufficient to increase yield of the monomeric form of IFN-β as compared to the yield obtained in the absence of arginine or sodium chloride in the pharmaceutically acceptable buffer. For arginine, the amount sufficient to increase yield is about 0.2 M to about 1.0 M, preferably about 0.4 M to about 0.8 M, including about 0.4 M, 0.5 M, 0.6 M, 0.7 M, and 0.8 M. In one embodiment, the amount of arginine present in the pharmaceutically acceptable buffer is about 0.5 M. For sodium chloride, the amount sufficient to increase yield is about 0.2 M to about 1.2 M, preferably about 0.2 M to about 1.0 M, more preferably about 0.5 M to about 1.0 M. In one embodiment, the amount of sodium chloride present in the pharmaceutically acceptable buffer is about 1.0 M.

The second purification method for preparing a composition comprising substantially monomeric IFN-β is similar to the first method, but provides a means of preparing this composition without the precipitation step. This second method comprises two basic steps: (1) mixing a sample comprising substantially purified IFN-β with guanidine hydrochloride (HCl) to obtain a solution comprising solubilized denatured IFN-β; and (2) renaturation of the IFN-β, preferably via dilution using an acceptable buffer. The guanidine HCl serves as a solubility enhancing agent as noted

Following the diafiltration or dialysis step with a pharmaceutically acceptable buffer of choice to remove residual guanidine HCl, the resulting pharmaceutical compositions may be stabilized against denaturation and loss of biological activity by the inclusion of a stabilizer in the pharmaceutical compositions, which includes but is not limited to proteins or carbohydrates, preferably chosen from the group consisting of mannitol, sorbitol, glycerol, dextrose, sucrose, and trehalose, or a mixture thereof. In a further aspect of the present invention, the IFN-β preparation obtained from the diafiltration (or dialysis) and stabilization steps may be lyophilized and reconstituted in an inert, non-toxic, physiologically compatible carrier medium for therapeutic and clinical applications.

The pharmaceutical compositions of the invention are formulated with a known concentration of the substantially monomeric form of IFN-β such that administration of a particular dose promotes a desired therapeutic response with respect to a particular IFN-β responsive condition undergoing therapy. By "desired therapeutic response" is intended an improvement in the condition or in the symptoms associated with the condition.

Pharmaceutical compositions comprising the IFN-β are useful in therapy directed to treatment of IFN-β responsive conditions. By "therapy" is intended treatment of an existing normal condition that is enhanced by IFN-β therapy, therapeutic treatment of an abnormal condition that is responsive to IFN-β, and preventive or prophylactic procedures comprising treatment with IFN-β so as to prevent or lessen the severity of an occurrence of an abnormal condition. By "IFN-β-responsive condition" is intended any condition that responds either positively or negatively to IFN-β. Such an IFN-β-responsive condition may be a normal condition. For example, a mammal may undergo IFN-β therapy to increase the responsiveness and/or capability of the immune response. Such therapies encompass treatment to provide protection against or modulate the severity of viral infections, for example, Dengue virus or Sindbis virus. In contrast, the IFN-β-responsive condition may be an abnormal condition such as malignant melanoma. Such abnormal conditions may be chronic, and thus occur more or less continuously, or such abnormal conditions may be acute. The IFN-β-responsive condition might be a condition which could possibly be characterized as both chronic and acute, such as remitting-relapsing multiple sclerosis. Any IFN-β-responsive disorder may benefit from administration of the

chromatograms in Figure 1 show that although non-covalent multimers were obtained at lower pHs, monomeric interferon-beta was obtained at pH 5.0 and above.

TABLE 1: Relative Yield After Dilution of Guanidine HCl (8M) IFN (~10 mg/ml) Estimated by HPLC

		Guanidine HCl Concentration After Dilution			
10 mM Buffer	**pH**	**0.2 M**	**0.4 M**	**0.8 M**	**1.6 M**
Glycine	3	2	2.1	1	1
Sodium acetate	4	13	12.7	6	2
Sodium acetate	5	68	68	33	30
Sodium citrate	6	79	62	43	42
Sodium phosphate	7	82	71	41	48
Tris HCl	8	99	83	40	38

TABLE 2: Percent Aggregates After Dilution of Guanidine HCl (8M) IFN (~10 mg/ml) Determined by HPLC

		Guanidine HCl Concentration After Dilution			
10 mM Buffer	**pH**	**0.2 M**	**0.4 M**	**0.8 M**	**1.6 M**
Glycine	3	78	51	35	39
Sodium acetate	4	<1	3	4	11
Sodium acetate	5	1	1	1	2
Sodium citrate	6	1	1	1	2
Sodium phosphate	7	1	1	1	3
Tris HCl	8	1	2	1	2

Example 3: Yield of Guanidine Dilution Step

The process was scaled up to evaluate the yield of the guanidine dilution step. The results in Table 3 show that a 41% to 57% yield can be obtained with a forty-fold dilution at pH 6 to 8 with a final protein concentration of about 0.15 mg/ml. SDS concentrations in samples tested were less than 10 micrograms per milligram of IFN.

TABLE 3: Refolding Recovery from 8 M Guanidine HCl (40x dilution)

10 mM Buffer	**pH**	**[IFN] mg/ml**	**% Yield**
Sodium citrate	6	0.12	41
Sodium phosphate	7	0.17	57
Tris HCl	8	0.15	52

Example 4: Removal of Residual Guanidine HCl Present After Dilution by Dialysis

Dialysis was used to remove residual guanidine HCl present after dilution. At pH 5, the highest yield, 83%, was obtained without additional NaCl present; and at

THAT WHICH IS CLAIMED IS:

1. A method for preparing an injectable formulation of interferon-beta (IFN-β) comprising:

a) preparing a first solution comprising IFN-β, isolating a pool of purified IFN-β from this solution, and precipitating said IFN-β from this pool using an alcohol to form a precipitate;

b) dissolving said precipitate in guanidine hydrochloride (HCl) to form a second solution comprising resolubilized denatured IFN-β and guanidine HCl;

c) diluting said second solution into a first buffer to obtain a third solution comprising resolubilized renatured IFN-beta and residual guanidine HCl; and

d) removing residual guanidine HCl from said third solution by diafiltration or dialysis of said third solution into a second buffer that is pharmaceutically acceptable, whereby said injectable formulation of IFN-β is prepared.

2. A pharmaceutical composition comprising substantially monomeric IFN-β produced by the method of claim 1.

3. The method of claim 1, wherein said second buffer contains arginine or sodium chloride.

4. The method of claim 1, wherein said first buffer has a pH of about 5.0 to about 8.0, and wherein said residual guanidine HCl is present in said third solution at a concentration of 1.6 M or less.

5. A method for preparing an injectable formulation of interferon-beta (IFN-β), said method comprising denaturation of IFN-β with guanidine hydrochloride (HCl) followed by renaturation of the IFN-β via dilution into a first buffer to obtain a renatured IFN-β solution comprising residual guanidine HCl, and removing said residual guanidine HCl from said renatured IFN-β solution by diafiltration or dialysis of said renatured IFN-β solution into a second buffer that is pharmaceutically acceptable, whereby said injectable formulation of IFN-β is prepared.

Figure 1
Dilution of Guanidine HCl (8 M) IFN (~10mg/ml) to 0.2 M Guanidine in Various Buffers Sizing HPLC Chromatograms

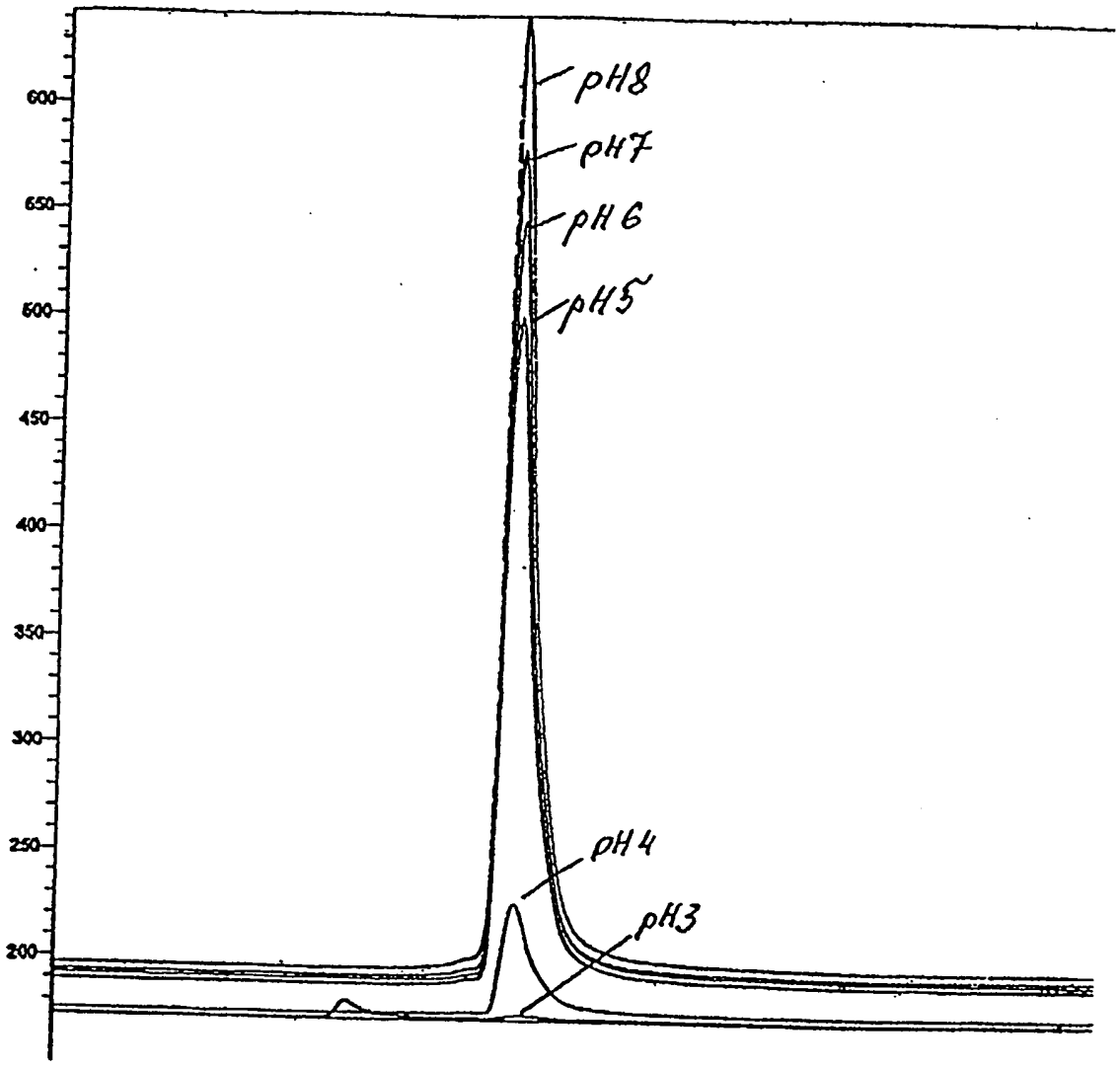

Figure 3
Effect of Tween 80 (% concentration) on the Aggregation of Renatured IFN